FINAL REPORT

Purifying and Testing Gecko Skin Compounds, a Promising Attractant for Small Brown Treesnakes

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Brown Treesnakes (Boiga irregularis) are an invasive pest on Guam and pose a severe threat to other islands. Attractants are particularly needed for small snakes, which are resistant to current control techniques. We extracted skin compounds from geckos (Hemidactylus frenatus), a preferred prey of the small snakes, and used bioassays to identify which of the successively more purified extract fractions contained compounds attractive to the snakes. Snakes responded to a non-fractionated gecko extract (rather than to controls). Several snakes also responded exclusively to matrices scented with one out of two fractions resulting from an extract separation step. Gas chromatography-mass spectrometry identified cholesterol as the dominant component in the bioactive fraction; other compounds (e.g., fatty acids and hydrocarbons) occurred in much smaller amounts. While the snake attraction effect of pure cholesterol needs to be demonstrated, as other minor compounds could elicit or enhance its attraction effect, our results demonstrate that it is possible to identify chemical components in fractionated gecko extracts that elicit a feeding response in Brown Treesnakes.

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APCI	atmospheric pressure chemical ionization
BTS	Brown Treesnake (*Boiga irregularis*)
CSU	Colorado State University
DNM	dead neonatal mouse/mice (*Mus domesticus*) (weight 1.2 – 2.2 g)
EtOAc	ethyl acetate
ESI	electrospray ionization
FT-ICR-MS	Fourier-transform ion cyclotron resonance mass spectrometer
GC-MS	gas chromatography-mass spectrometry
Hex	hexane
LC-MS	liquid chromatography-mass spectrometry
MeOH	methanol
MS	mass spectrometry
Si-Hex	silica column separated hexane fraction
Si-Hex-DCM	silica column separated hexane/dichloromethane fraction
Si-DCM-MeOH	silica column separated dichloromethane/methanol fraction
SVL	snout-vent length (a snake's length excluding the tail)
USGS	U.S. Geological Survey

Keywords Attractant, bait, *Boiga irregularis*, Brown Treesnake, chemistry, compounds, gecko, Guam, invasive, juvenile, snake

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Abstract

Objectives: To identify one or more chemical compounds that could serve as an attractant to invasive Brown Treesnakes (*Boiga irregularis*) smaller than 700 mm snout-vent length.

Technical Approach:We extracted skin compounds from geckos (*Hemidactylus frenatus*), a preferred prey of small Brown Treesnakes, and used bioassays to identify which of the successively more purified extract fractions contained compounds attractive to the snakes. Chemical analyses followed to characterize compounds in fractions of interest. For the study to be classified as a success we wanted to identify one or a few dominant compounds in a fraction that had a clear and consistent attraction effect on the snakes; this (these) compound(s) would be strong candidates for snake attractants if they are commercially available or possible to synthesize in a cost-effective manner.

Results: Many snakes did not respond to either treatments or controls. Of those snakes that did respond, the majority responded to the treatment with gecko scent rather than to controls. In one experiment, all snakes that responded did so to a crude, non-fractionated gecko extract; they ingested or chewed on pieces of eraser scented with the extract (but not un-scented control erasers). In another experiment, several snakes responded exclusively to erasers scented with one out of two fractions resulting from a first extract separation step. However, after separating the bioactive fraction one step further, we detected no attraction to any of the three resulting sub-fractions. Gas chromatography-mass spectrometry identified cholesterol as the dominant component in the bioactive fraction besides other compounds (e.g., fatty acids and hydrocarbons), which occurred in much smaller amounts. The snake attraction effect of pure cholesterol has yet to be verified, and we do not know if addition of other compounds would enhance its attraction effect, but our results provide a proof of concept for extraction, purification, testing, and analysis of chemical compounds acting as attractants to small Brown Treesnakes. Cholesterol is commercially available at a low cost.

Benefits: Finding a non-animate attractant for the Brown Treesnake—a costly invasive pest that has devastated Guam's avifauna and poses a severe threat to other islands—would greatly facilitate its containment in Guam as well as help eradicate it in Guam and any other locations it may show up. This is especially true for attractants suitable for small snakes, as they have proven very resistant to the current rodent-based control techniques. Their favored gecko prey is logistically difficult to use in large-scale operational control. Finding a cheap, non-animate compound that could help lure small snakes into traps, or be used to scent matrices for oral toxicants, would be a major step forward for Brown Treesnake control. Our results demonstrate that it is possible to identify chemical components in fractionated gecko extracts that elicit a feeding response in Brown Treesnakes. Further research and development would need to involve identification of compounds that occurred in minor amounts, and screening for snake attraction efficacy of cholesterol as well as mixes of compounds.

Objective

The goal of this study was to identify one or a few chemical compounds that attract small (less than 700 mm snout-vent length) Brown Treesnakes (*Boiga irregularis*). Criteria for success include attraction of small snakes to the extract and qualitative characterization of the substances. These small snakes have proven very difficult to control with the methods used in operational, large-scale snake control on Guam, and logistics (and/or cost-effectiveness) prevent their favored gecko prey from being used for snake control on a large scale. If we could find a chemical compound (or possibly a cocktail of a few chemicals) that attracts small snakes and is either commercially available or can be synthesized in a cost-effective manner, the opportunities to control small Brown Treesnakes would increase dramatically. We therefore aimed at extracting gecko skin compounds and presenting them to small Brown Treesnakes in laboratory bioassays, using the crude extract as well as fractions from successive purification steps. We planned to investigate the number of, the relative quantities of, and the qualities of chemical compounds present in any bioactive fraction(s).

Background

The Brown Treesnake (*Boiga irregularis*, BTS) was accidentally transported to Guam at the end of WWII, presumably with military cargo, and a few decades later it had spread across the entire island (Savidge 1987, Rodda et al. 1992). When the population peaked, densities reached up to ca 100 snakes / ha (Rodda et al. 1992). It proved to be a costly invasive pest, causing numerous power interruptions, extinction of the majority of Guam's forest birds, and human envenomations (Rodda and Savidge 2007). Since Guam is a transport hub for the Western Pacific and has a high military presence, transport of people and goods by sea and air now threaten to spread the snake to new locations and cause similar havoc elsewhere. For example, should the snake get established in Hawaii the potential annual damage may exceed $1 billion (Shwiff et al. 2010). A BTS control program is therefore in place on Guam, the current main objective of which is to contain the snakes (Vice and Pitzler 2002).

The Brown Treesnake control program in Guam uses two main methods for containment: snake removal around seaports, airports, and cargo facilities; and searching vessels and cargo using specially trained canine teams (Engeman and Vice 2001, Vice and Pitzler 2002). Control efforts rely primarily on traps baited with a live mouse (Vice et al. 2005). Trials have also been conducted with an oral toxicant (acetaminophen) inserted in a dead bait mouse that is placed in a bait station (Savarie et al. 2001). Unfortunately, traps are ineffective in targeting snakes smaller than ca 700 mm snout-vent length (SVL), and BTS have to reach approximately 900 mm to become fully susceptible to trapping (Rodda et al. 2007, Tyrrell et al. 2009). Our recent research (B. Lardner et al. *unpublished data*) suggests small snakes are also refractory to control using dead rodents, and that using smaller (neonatal) rodents will not affect this size-threshold to any significant extent. While visual searches are useful for detecting small snakes (Rodda et al. 2007, Christy et al. 2010), they are a very labor intensive method that would require more or less permanent surveillance of the entire perimeter of focal facilities. As a consequence, small snakes can easily seep through the first line of defense – traps on fences – and small snakes are therefore at particular risk of entering cargo (Vice and Vice 2004). This leaves the canine search teams as a last line of defense against snakes departing Guam. In blind tests, canine teams detect 35-70% of snakes present in cargo (Vice and Pitzler 2002). We also know that the cohort of snakes <800 mm SVL may occasionally make up as much as half of the population at any given place and time (G. H. Rodda et al. *unpublished data*; S. R. Siers et al. *unpublished data*; see also Rodda et al. 1999a). Adding these facts together, it is not surprising that BTS, assumed to have originated from Guam, have been found in several locations across the Pacific, in the U.S. mainland, Indian Ocean, and as far away as Spain (Stanford and Rodda 2007). Many of these records are associated with military transports and military facilities (Fritts et al. 1999). This calls for the development of a means to control small snakes that are not possible to target effectively with the existing rodent-based control methods.

Small Brown Treesnakes prefer geckos over mice (Lardner et al. 2009), and snakes trapped with gecko bait are, on average, smaller than those trapped with mouse bait (Rodda et al. 1999b). It therefore seems reasonable to assume that an attractant based on their preferred gecko prey would offer the best option. The overall efficacy of gecko-baited traps has however been low (Rodda et al. 1999b, B. Lardner et al. *unpublished data*), and there may be reasons other than prey preferences contributing to the poor trapping results for small individuals. The USGS-CSU Brown Treesnake Project is currently investigating alternative and/or contributing hypotheses regarding the movements and hunting strategies of small snakes. Gecko-based control could either utilize live geckos as lure in traps, dead geckos as vectors for oral toxicants, or use an artificial (non-animate) scent attractant that mimics the scent of geckos.

Unfortunately, methods requiring live geckos are logistically challenging to use in large-scale operational control, as geckos cannot be bred in large numbers in a cost-effective manner. Geckos have a relatively low intrinsic rate of increase compared to rodents, partially because of a small clutch size (normally, two eggs are laid at a time) and partially because they take longer to reach sexual maturity [67 – 283 days in *Hemidactylus mabouia* (Klowden 2007) compared to 42 – 49 d in domestic mice (Gerlach 1998)]. Also, most geckos feed on invertebrates and providing a breeding colony with food is much more challenging (and costly) than feeding a rodent colony. Collecting wild geckos in quantities required for a long-term and/or large-scale snake control program is also very challenging from a logistic perspective, even at a location such as Guam where non-native geckos occur at very high densities. On Guam, the Asian House Gecko (*Hemidactylus frenatus*) can sometimes reach densities of 3,000 individuals / ha in forested habitats (Rodda et al. 2005); yet semi-professional gecko collectors targeting suitable urban habitats with high gecko population densities sometimes have difficulties catching more than 50 individuals in an evening (B. Lardner, *pers. obs.*). They also face a risk of locally overexploiting the populations, should there be a steady demand for more geckos. In addition, some may argue that ethical considerations of using live animals as lure, or killing them for scent production or dead bait, calls for the development of a non-animate attractant. Hence, finding a cheap, non-animate compound that would help lure small snakes into traps, or be used to scent matrices for toxicants, would be a major leap forward for BTS control.

Until 2008, no research had been done on developing a gecko-based attractant. At that time we conducted a pilot study where we used a mix of three solvents (hexane, methanol, and ethyl acetate) to extract the scent of geckos (*Hemidactylus frenatus*) (Fig. 1). In a laboratory feeding trial, we simultaneously offered three types of dead neonatal mice to small snakes: one un-manipulated control, one control tainted with solvents, and one mouse tainted with the gecko extract. While five snakes would not accept any of the mice (but readily took a dead gecko after the trial), all twelve snakes that ate any bait took the gecko-scented mouse first. When the snakes were repeatedly subjected to the same trial this preference became less clear, suggesting that by ingestion of gecko-scented mice, the snakes may gradually 'learn' to feed on un-scented mice.

Figure 1. The Asian House Gecko (*Hemidactylus frenatus*) is introduced to Guam. With population densities sometimes as high as 3,000 per hectare, it constitutes an important food source for juvenile Brown Treesnakes.

Materials and Methods

Our general approach was to extract scent compounds present in the skin of geckos and present captive BTS to more and more purified extract fractions, thereby letting the snakes guide us to what fractions might contain compounds of interest.

The methods for extractions and bioassays changed over the course of the project, as we came to realize that some aspects of the anticipated methods did not produce sufficiently strong results (or no useable results at all). However, there were aspects in common for all phases. We here outline the general methods and the details for each consecutive study phase, referred to as Phases 1; 2A, B; 3A, B; and 4A, B (Table 1, p. 12).

Gecko Scent Extraction

Asian House Geckos (*Hemidactylus frenatus*) used for scent extraction were collected in Guam and euthanized with carbon dioxide (CO_2) the morning after they were collected. In Phase 1 we froze and stockpiled geckos before extraction, but for Phase 2 and onwards we extracted freshly euthanized geckos. For a "crude" extract (i.e., before any separations were conducted), a number of euthanized geckos were submerged completely in a capped vial with a mixture of two or three different organic solvents in equal amounts (in Phase 1, methanol and ethyl acetate; from Phase 2 and onwards we used a mix of methanol, ethyl acetate and hexane). We subjected the vial to a 1-minute ultrasound bath, and then left the geckos sitting in the solvent blend for another 30 minutes during which the vial was spun occasionally. We then removed the geckos (Fig. 2), capped, and stored the extract vials in a freezer until used.

Scent Extract Separations

With the exception of Phase 2, where we scented baits with reconstituted (in methanol and ethyl acetate) crude extract containing all extracted compounds, we tested fractions of the crude extract for snake-attraction effects. In a liquid-liquid separation step we separated the crude extract (which previously had been filtered and dried-down in a rotary evaporator) with a mixture of hexane and methanol. The resulting fractions – compounds in the hexane layer versus those in the methanol layer – were dried down in pre-weighed vials in a speed-vac concentrator, allowing us to calculate the amount of dry matter they rendered; they were then re-constituted with the respective solvent (hexane or methanol). These fractions were tested in Phase 1 and Phase 3.

Figure 2. Chemical compounds present on the surface of dead geckos were extracted by immersing them in a mix of organic solvents.

For Phase 4 we used the bioactive fraction from Phase 3 as a basis for further separation. We subjected the bioactive fraction to a column separation over a silica matrix using pure hexane, followed by a 50/50 mix of hexane and ethyl acetate, and finally rinsing the column with pure ethyl acetate; rendering us three fractions. The third (pure ethyl acetate) fraction contained only a very small amount of dissolved matter (as measured from its dried mass) whereas the first two fractions – henceforth referred to as the Hex and Hex-EtOAc fractions – occurred in much larger amounts and similar dry masses. We considered it desirable to taint the bait with equal amounts of compounds, in terms of dry weight, so we opted to test the first two (Hex and Hex-EtOAc) fractions against each other, and test for a snake attraction to the EtOAc fraction separately. We reconstituted the dried compounds in the same solvent/s with which they were eluted from the column.

Scent Dosages

The number of geckos required to produce a given weight of extract depends on their size; the larger the gecko, the larger the surface area from which skin compounds are dissolved by the solvents. For this study we were less focused on the number of geckos it took to produce a certain dry weight of extract because the dry weight of extract from one "standard" (in terms of size) gecko will become less and less the more it is fractioned (and as non-bioactive fractions are discarded). Instead, our focus was on the amount of dry weight equivalents we applied to each bait matrix offered to snakes in the bioassays.

In the separation for Phase 1 we obtained a reasonably balanced amount (weight) of dried extract from the two fractions (23.2 mg hexane- and 27.5 mg methanol-fraction, respectively). We chose to resolve them in equal amounts of solvents and then apply an equal volume to the matrices in the two treatments. This means we applied slightly different dry weights to the two scent treatment matrices (0.46 mg dry mass for the hexane fraction treatment; 0.55 mg dry mass for the methanol fraction treatment; Table 1). In Phase 2 where we had only one gecko-scented matrix scented with an un-separated solution (and two types of control matrices), we used a dosage of 4 mg. In Phases 3 and 4A we applied the same dry weight (2 mg) to both scented matrices in a trial. We obtained such minute amounts from the ethyl acetate fraction in Phase 4 that we ended up applying <1 mg to the scented matrix in Phase 4B, where we used one control matrix. This was a chosen trade-off between a small sample size (but a larger amount of compounds applied to each scented bait matrix) on one hand, and a very small amount of compounds applied to each scented bait matrix (but a large sample size) on the other hand. However, as explained in the beginning of this sub-section, even this minute amount could theoretically elicit a very strong response in the snakes, provided that this ethyl acetate fraction was the *only* fraction containing a bioactive compound.

Figure 3. Field assistant scrutinizing a Brown Treesnake that was too large to be used in this study.

Snake Bioassays

From April – November 2010, we collected Brown Treesnakes <700 mm SVL (and a few that later proved to be slightly longer) through visual searches at night at various sites in northern Guam (Fig. 3). The morning after a snake was collected it was brought to the lab, measured and weighed, and housed in a cage that would later double as a test arena during the bioassay. The cage was a white 5-gallon bucket with wire mesh-covered ventilation panels (total area ca 0.01 m^2) near the bottom. A glass plate was used as a lid. The cage was lined with paper (for easy cleaning), fitted with a plastic water bowl that doubled as a hide box (a hole was cut in a side allowing the snake to get under it), and we placed a few twigs in the cage for a substrate that the snake could climb on. The cage was misted lightly with water every 2 – 3 days. To ensure snakes were motivated to forage (and respond to scent cues we presented in a bioassay ca 1 week after their capture), they were not fed prior to their testing. The snakes were housed (Fig. 4) and tested in a room with ambient temperature (ca 29°C throughout the day and night). We clock-shifted the 12:12-hour light cycle so that the lights went off at noon and on at midnight.

Figure 4. Snakes were individually housed and tested in 5-gallon buckets with a glass lid that allowed us to record their behavior at night using surveillance cameras and infrared illumination. In this photo, no bait matrix dishes are in the cages; those were added to the buckets of snakes to be tested immediately before a trial started.

We fitted three 2-cm sections of hose to the wall of the bucket, 50 mm from the upper rim. These later allowed us to suspend a plastic Petri dish (diameter 148 mm) fitted with three wire legs close to the lid. The dish served as a tray on which we simultaneously offered three (in Phase 4B only two) alternative bait matrices during a trial. Because the bait matrices looked identical, we attached small pieces of paper with symbols to the legs next to the dish. Differently treated bait matrices were placed on the dish adjacent to specific symbols, thereby allowing us to see from surveillance video which of the bait matrices a snake interacted with (Fig. 5).

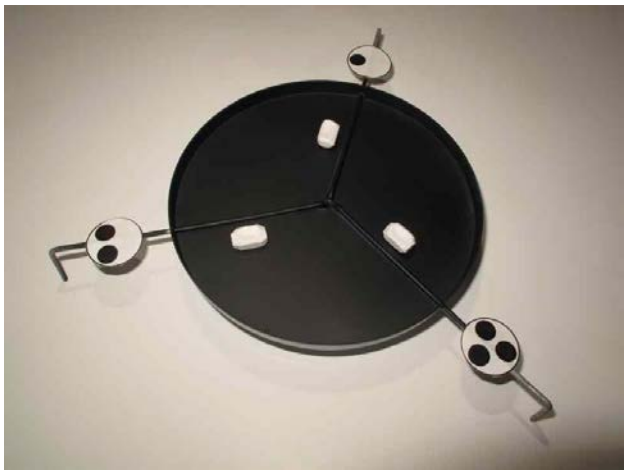

Figure 5. Differently scented bait matrices (or un-scented controls) were simultaneously offered to snakes on a Petri dish that was suspended in the snake's cage 5 cm below the glass lid covering the cage. Because the bait matrices looked identical, we aligned them with the treatment-associated markers seen attached to the legs. This allowed us to determine from surveillance video (in infrared light) which of the bait matrices a snake interacted with.

In a small-scale pilot study (conducted in 2008) we had used dead neonatal mice (DNM) as a bait matrix. While many (but not all) snakes <700 mm SVL will eat neonatal mice in a captive situation, we found that they investigated the mice before choosing which to first consume, and they chose to start the meal with the mouse scented with a crude gecko extract (after which several snakes continued consuming one or two more mice that were not gecko-scented). In phase 1 of this study, we therefore used DNM (weight range 1.2 – 2.2 g; size-matched to within ±0.1 g in each snake trial) as a bait matrix, and used the *first* item ingested as an indicator of which scent treatment snakes preferred. We continued doing this in Phase 2A, but the snakes proved less choosy and the results less clear than we had expected. With a weak signal-to-noise ratio, we faced an unexpected high demand for snakes. That was especially so as we wanted to avoid using a snake for more than one trial; given the results from the pilot study, we were concerned that snakes which had been tested repeatedly would learn to take neonatal mice (a food source they had probably never encountered before). In Phases 2B, 3B, and 4A, B, we therefore opted for a bait matrix that – unless scented – should be ignored by the snakes, yet have a size, shape, and texture that could pass for something edible, should it smell right. We choose erasers - regular, white office stationary eraser blocks (Staedtler® "Mars plastic" latex free erasers; Table 1). We cut the erasers to rectangular blocks roughly the size of a lizard (e.g., *Carlia ailanpalai*) egg, rounded the edges, and cut a depression on one side where we could drip

scent solution or – for a control treatment – a blank solvent mix (Fig. 6). The weight of the eraser blocks varied between 0.8 g and 1.7 g over the course of the study, but the three matrices (in phase 4B only two matrices) offered to a snake in a trial were always size-matched. These blocks were small enough to be ingestible by the snakes they were offered to, and we tried to offer the smaller snakes the smaller erasers.

Figure 6. To apply a standardized and known amount of scent to each bait matrix (and to produce the appropriate un-scented controls), we applied scent solution of a known concentration (or equal amounts of "blank" solvent(s) for the control treatment) with syringes. To avoid that a clouded extract was more concentrated for the first than for the last bait matrices to be scented, we shook the syringe and applied a small drop to all matrices to be scented with that extract (all matrices in one of the three rows seen in the figure); let the solvent evaporate; then shook the syringe and applied another round of drops to all matrices; and so on. The solvent had evaporated by the time we presented the matrices to the snakes in the bioassays (normally about an hour after finishing the scenting of the matrices).

Our goal was to test each snake only once. However, as suitably-sized snakes were very difficult to obtain in large numbers, we were forced to make exceptions to this rule. First, snakes that did not come out from their hide during a trial were considered un-tested and subjected to a new trial on another day. Second, snakes that showed no interest in the bait offered (but often seemed more interested in finding a way to exit the cage) were often tested again. The latter was especially the case in Phase 3B (using eraser matrices), where several snakes that showed no interest in any eraser were tested again with neonatal mice as the matrix (Phase 3A). We did not, however, test any snake again if it had shown a behavior that was considered a choice of any kind, thereby avoiding pseudoreplication.

For most of the study we restricted the video-monitored trial to 2 hours immediately after the lights in the snake room went out. This coincides with the time snakes were most active. It appeared they were often active for an hour or two before realizing there was no way out of the cage and nothing (more) to eat, after which they went back into their hide and (apparently) remained inactive for the reminder of the night. We removed the bait matrix dish after the end of the 2-hour trial. Towards the end of the study we allowed snakes access to the baits throughout the 12-hour night phase. This gave us a chance to see if they took any bait even if they did not emerge from the hide during the first two hours after the lights were turned off. However, snakes that interacted with bait normally did so in the first hour after emergence from the hide.

We scored snake behavior and interactions with the bait from recorded surveillance videos. For a trial with mouse bait to qualify as valid, the snake had to be out and active, and encounter the dish with the baits. For the first bait ingestion to qualify as a valid and fully informed choice, the snake had to pass with its head immediately next to (within tongue-flick range of) all three mice before it started to ingest a mouse. Trials where the snake ingested any bait before encountering all three baits were discarded (and the snake euthanized). Admittedly, with a large enough sample size, inclusion of such uninformed choices could indeed help to show if there was any preference for one particular bait treatment. However, our sample sizes were limited and we chose to avoid analytical noise by excluding these uninformed bait takes.

Many Phase 3B-trials (with eraser bait) where the snake was not out and active were repeated on another day. We screened videos for any indication of snake interest in the eraser baits, and took notes on snakes biting or chewing any of the baits as well as any bait ingested.

After a snake had completed the bioassay trials, we measured it again before euthanizing it.

An overview of some key features of the different experimental phases is given in Table (1).

Table 1. Key features of the different experimental phases. Solvents and fractions are abbreviated as Hex = hexane, MeOH = methanol, EtOAc = ethyl acetate; DNM = dead neonatal mice. This is not an exhaustive list of features; see the text for additional information.

Phase #	Bait Treatments Offered to Snakes	Bait matrix	Dosage[a] (mg)
1	Liquid-liquid separated fractions from crude extract (Hex vs. MeOH vs. control)	DNM	0.46; 0.55; (0)
2A	Crude extract vs. two different controls	DNM	4.0; (0); (0)
2B	Crude extract vs. two different controls	eraser blocks	4.0; (0); (0)
3A	Liquid-liquid separated fractions from crude extract (Hex vs. MeOH vs. control)	DNM	2.0; 2.0; (0)
3B	Liquid-liquid separated fractions from crude extract (Hex vs. MeOH vs. control)	eraser blocks	2.0; 2.0; (0)
4A	Column separated fractions from the Hexane fraction of Phase 3 (Hex vs. 50:50 Hex:EtOAc vs. control)	eraser blocks	2.0; 2.0; (0)
4B	Column separated fraction from the Hexane fraction of Phase 3 (EtOAc vs. control)	eraser blocks	<1.0; (0)

[a]Dosages are listed for treatments specified under "Bait treatments offered to snakes". Controls are indicated as (0).

Chemical Analyses of Scent Extracts

Because of the paucity of snake responses in the Phase 4 bioassays, we focused on the fractions used in Phase 3 for our chemical analyses. To get the most information about the composition of the fractions, two analytical procedures were used: gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS).

We conducted GC-MS experiments to determine the complexity of the hexane fraction used in bioassay phases 3A and 3B. Besides analysis of that fraction, we also analyzed three sub-fractions thereof, which had been obtained by another fractionation step. For the fractionation we used 5 mg of hexane extract and loaded it onto a silica column and sequentially eluted it with 30 ml hexane, 40 ml hexane/dichloromethane (9:1), and 50 ml dichloromethane/methanol (9:1). After drying off the solvents we received 0.39 mg hexane sub-fraction (Si-Hex), 0.11 mg

hexane/dichloromethane (Si-Hex-DCM) and 4.32 mg dichloromethane/methanol (Si-DCM-MeOH) extract. The hexane and each sub-fraction were derivatized using trimethylsilyl to obtain trimethylsiloxy groups in the test compounds, making them more volatile for the GC-MS analysis.

Fractions were injected into the GC-MS for analysis. Like with all chromatographic techniques, separation of compounds was achieved by reaching equilibrium between the mobile phase (in gas chromatography, e.g., nitrogen, helium) and the stationary phase (a thin layer of the separation column, which is usually gradually heated). Thus, using GC requires analytes to be volatile and heating of the stationary phase accelerates the rate with which equilibrium is reached. Depending on the compounds' affinity to the mobile and/or stationary phase, the compounds elute earlier (i.e., at lower temperatures) or later (at higher temperatures) from the stationary phase. After injecting the sample, a gas stream (e.g., nitrogen, helium) took the mix of compounds down a long separation column. As the compounds came off the column they were captured, ionized and detected by the mass detector. Thus, information on the identity of compounds could be gathered by the retention time (i.e., the time it takes for them to elute from the column) and, primarily, by the mass detected. To verify the identity of certain molecules we fragmented compounds into compound pieces in the mass spectrometer and measured the masses of these fragments (GC-MS/MS). This allowed further identification of molecules with similar retention time and mass, as the fragments are largely compound specific.

Prior to LC-MS analysis we purified the methanol sample to remove any insoluble remains and possible salts as they would interfere with and potentially clog the ultrahigh-resolution mass spectrometer used for the LC-MS analyses (Bruker Solarex, 15 Tesla Fourier-transform ion cyclotron resonance mass spectrometer, FT-ICR-MS). The purification involved loading the methanol extract on solid phase extraction columns, washing it with omnipure water to remove any salts, and final elution of the methanol soluble compounds via rinsing with methanol. The hexane fraction used in the LC-MS was not further purified, since salts cause mainly problems in the more polar methanol fraction. Both methanol and hexane fractions were dissolved in the respective solvents (MS-analytical grade methanol and hexane). Each sample was injected directly into the FT-ICR mass spectrometer, where the individual compounds of the fraction (hexane or methanol) were ionized (if the compounds were amenable to ionization in this particular ionization mode). The methanol fraction was analyzed with electrospray ionization (ESI negative mode), while the hexane fraction was analyzed with atmospheric pressure chemical ionization (APCI negative mode), to adjust analysis conditions to the different solvent systems of the samples. This resulted in hundreds of mass peaks of variable intensity, the intensity providing information on how much compound was present in the injected sample (assuming the compound ionized). Since the FT-ICR mass spectrometer is exceptionally sensitive, it provides high-resolution mass data for the detected peaks. Based on the high resolution mass, the molecular formula can be calculated by the analytical software of the machine (Bruker Solarex). In short, the measured mass is divided by the exact mass of possible

elements (e.g., C, H, O) and multiples thereof until the calculated mass matches the measured high resolution mass. As we were interested only in the major compounds of each fraction, we set the intensity threshold for analysis rather high to limit the number of peaks. This still resulted in about 100 peaks in the methanol and hexane fractions that were compared in a cross table with each other to identify peaks present only in the hexane fraction (active with BTS in the feeding trials; see appendix for list of peaks).

Results and Discussion

Snake Bioassays

Phase 1

In this phase we used DNM as the bait matrix. Three DNM were offered simultaneously to a snake in a trial. One was tainted with a solvent control (a mix of hexane and methanol); another with the hexane fraction from a liquid-liquid separation; and a third with the methanol fraction from the same liquid-liquid separation. The extraction preceding the separation yielded only a small amount of crude matter, which was evident from its near lack of color. We applied ca 0.46 mg of dry matter to the hexane-treatment DNM versus 0.55 mg dry matter to the methanol-treatment DNM.

We conducted eight bioassays with as many snakes, measuring 523 – 702 mm (mean = 633 mm) SVL. One of the snakes struck and ingested a DNM before it had sampled all three items, and was therefore considered not to have made an informed choice. Of the remaining seven snakes, three (43%) took the control bait first, three (43%) took the hexane fraction treated bait first, and one (14%) took the methanol fraction treated bait first.

While admittedly a very small sample, the lack of a clear signal and the apparent low amount of dissolved compounds in the crude extract caused us to step back and proceed with Phase 2.

Phase 2

Trials used a crude extract made from freshly killed (not previously frozen) geckos that were immersed in a mix of hexane, methanol, and ethyl acetate. The reconstituted extract was tainted on a bait matrix (ca 4.0 mg dry matter / matrix), tested against two control bait matrices – one tainted with a mix of methanol and ethyl acetate (without any gecko involvement), the other a 'plain' bait matrix not tainted by anything. As a matrix we used both DNM (Phase 2A) and eraser blocks (Phase 2B). Snakes that did not respond to (i.e., ingest or bite) any of the baits offered in a trial were often tested again on a later date to rule out that their lack of interest was not a temporary effect of imminent shedding or some other unknown factor. Such repeated testing could occur with only one type of bait matrix (repeat with the same type of bait as in a previous trial) or with the other type of bait. However, as soon as a snake had made a choice (as scored from video or by the overnight ingestion of a bait matrix), it was retired from further use in the study.

In Phase 2A we conducted 65 trials using 55 snake individuals (range 442 - 710 mm SVL; mean = 624 mm). However, only 28 trials contributed informative and unequivocal results. Discarded trials included those where the snake took a mouse before sampling all three mice (and thus the choice could not be considered 'informed'); we also discarded any prior and inconclusive trials a

snake might have been subjected to and, as a consequence, never analyzed more than one trial per snake individual.

Of the 28 valid trials, 3 snakes (11%) were out and active during the trial but did not take any of the offered mice. To test if these snakes were at all interested in foraging, they were offered a dead *Hemidactylus frenatus* gecko after the trial. All three snakes ingested the gecko.

Of the remaining 25 snakes that ingested one or more mice during a trial, 6 (24%) ingested the solvent-tainted control treatment mouse first; 8 (32%) ingested the un-tainted control treatment mouse first; and 11 (44%) ingested the gecko-extract tainted mouse first.

In Phase 2B we conducted 34 trials with 18 snake individuals (range 556 – 699 mm SVL; mean = 642 mm), using eraser bait matrices. Most individuals were tested more than once (after not taking any bait in a previous trial), but we only analyzed one trial per snake. Of the 18 snakes, 11 (61%) did not ingest any of the baits, whereas 7 (39%) ingested the gecko-extract tainted bait. Three of them did so during the two hours of video-monitored trials; three snakes were not outside their hide during this period but came out and ingested the extract-tainted bait later at night. The video showed one snake active during the filmed trial, but it did not take the extract-tainted bait until later at night. No snake ingested any of the control treatment baits; nor were they seen chewing on them.

Taken together, Phase 2A and 2B (especially the latter) indicated that snakes were attracted by the crude, un-separated gecko extract. We therefore proceeded with Phase 3, now using the same crude extraction method as for Phase 2.

Phase 3

Trials used the hexane versus methanol fractions from a liquid-liquid separation based on crude extract made from freshly killed (not previously frozen) geckos, tested against a control (bait matrices tainted with a mix of hexane and methanol, without any gecko involvement). For each of the fraction-tainted treatments we applied an equivalent of ca 2.0 mg dry matter. We used DNM (Phase 3A) and eraser blocks (Phase 3B) as bait matrices.

Using DNM bait matrices we conducted a total of 52 trials with 45 snake individuals. Seventeen trials were discarded because either the snake ingested bait before encountering them all, or – in seven trials – the snake took no bait and the trial was repeated (then, the first of the trials was discarded). Of the 35 valid trials, using snakes between 377 and 695 mm SVL (mean = 485 mm), none of the three DNM was ingested in 24 trials (69%).

Of the remaining 11 trials, the solvent control-treated DNM was taken first in 3 trials (27%); the methanol-tainted DNM was taken first in 1 trial (9%); and the hexane-tainted DNM was taken first in 7 trials (64%). While the latter is a high figure (ca 2/3 as opposed to the expectation of

1/3 under a random choice), it is based on a small sample size. Furthermore, if we base the take rate on the full 35-trial sample, only 20% of the snakes indicated an attraction to the hexane fraction. The smaller snakes more often refused all of the mice offered than did the larger snakes (Wilcoxon rank sum test: P = 0.04; Fig. 7), but data are too sparse to address how snake size affected take rates of the different mouse treatments.

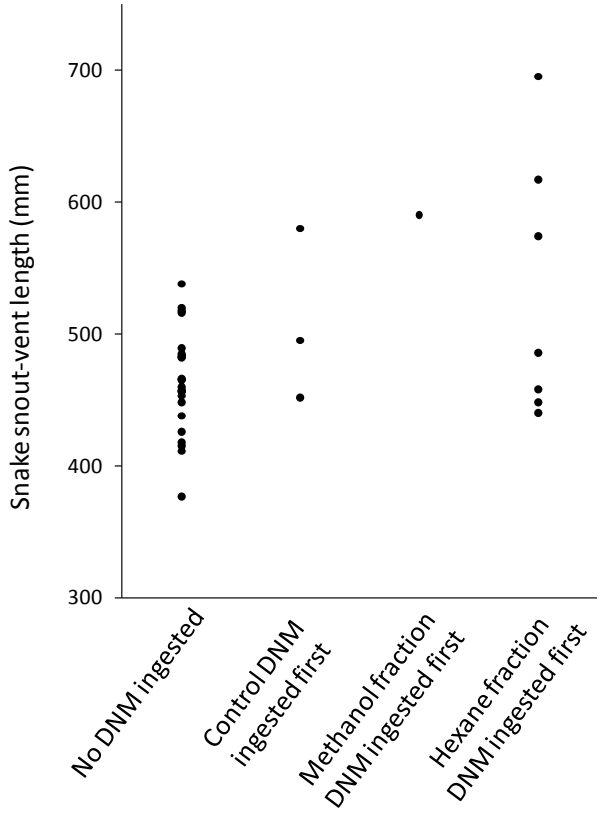

Figure 7. Size of snakes in Phase 3A in relation to their bait choices (or lack thereof) when using dead neonatal mice as the bait matrix.

Using eraser block bait matrices, we conducted a total of 92 trials with 66 snake individuals, ranging from 377 – 744 mm SVL (mean = 579 mm). Twenty-six trials were discarded because the snake was not active or the trial constituted a duplicate trial for the same snake (a trial when a snake did not make any choice and was later tested again).

Of the 66 valid trials (many of which were video-monitored in infrared light for the full 12-hr dark period), the snake ingested the hexane-tainted eraser matrix in five of the trials. In another eight trials the snake was seen chewing on the hexane-tainted eraser but releasing it. In another trial, the hexane-tainted eraser matrix was found on the bottom of the cage the following morning. It was not visible from the video if the snake chewed on it, but it seems unlikely that

the snake had managed to accidentally push the bait matrix over the 8-mm high lip of the prey presentation dish. However, we choose a conservative approach and only consider 13 snakes to have interacted with the hexane-extract tainted bait. No snake was seen to interact with (chew on) other baits, nor was any other bait type ingested by any snake. Hence, 13 out of 66 snakes (20%) responded positively to the hexane fraction (in a total of 92 *attempted* trials), whereas no snake was seen responding to the methanol treatment or the control treatment. Snakes responded

to the hexane fraction across the size range we tested (Wilcoxon rank sum test, contrasting snakes that did not interact with any bait against snakes that either chewed or ingested hexane fraction-tainted bait; $P = 0.37$; Fig. 8), but smaller snakes were prone to chew and release the tainted eraser baits whereas the larger snakes were more prone to ingest it (contrasting snakes that chewed hexane fraction-tainted bait against those that ingested it; $P < 0.01$).

Phase 4

This phase tested three different fractions derived from the hexane fraction that proved to attract snakes in Phase 3. One of the three fractions obtained from the column separation contained a considerably smaller amount of dissolved matter (as measured from its dry weight), so we chose to test (in Phase 4A) two of the fractions against each other (and against a solvent-only control); then test (in Phase 4B) the third (sparse) fraction on its own, against a solvent-only control. All trials used eraser blocks as the bait matrix; no DNM were used. Trials were screened by video for the first 2 hours. We then left the bait matrices in the cages for the remainder of the night to allow the snakes an opportunity to ingest them after the trials.

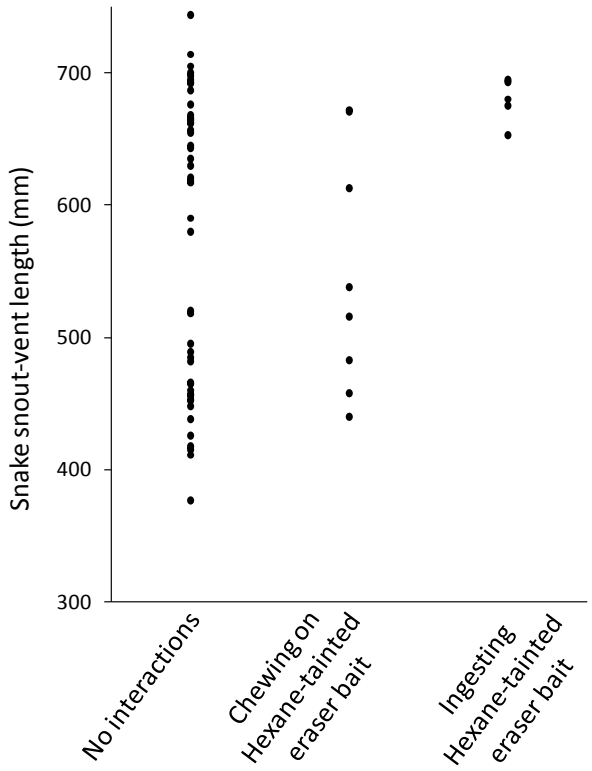

Figure 8. Size of snakes in Phase 3B in relation to their interactions with bait (or lack thereof) when using eraser blocks as the bait matrix. Interactions with methanol-tainted or control matrices are not shown because there were no such interactions.

In Phase 4A we attempted 36 trials with 36 snake individuals. All snakes but one were seen (from the recorded video) to be out and active during the 2-hr trial. The following morning we scrutinized the bait matrices on the dish of the snake that was not active; the bait matrices seemed to sit in exactly the same locations as they had been placed, indicating the snake had not been out (as baits are normally dislodged somewhat when a snake moves over the dish). Hence we assume the snake was not participating, and we focus on the trials where the snake was active. These 35 snakes measured 451 – 682 mm SVL (mean = 549 mm). None of the snakes were seen to chew on any of the erasers, and all erasers were still on the dishes the following morning. Hence, we registered no sign of any attraction to any of the treatments.

In Phase 4B we tested 16 snakes in one trial each. One of the snake trials was disqualified for the same reason as described above. In the remaining 15 trials, using snakes 436 – 553 mm SVL (mean = 489 mm), none of the snakes were seen to chew on any of the erasers, and all erasers were still on the dishes the following morning. Again, we registered no sign of any attraction to any of the treatments.

Figure (9) sums up the bioassay results from Phase 2 through 4.

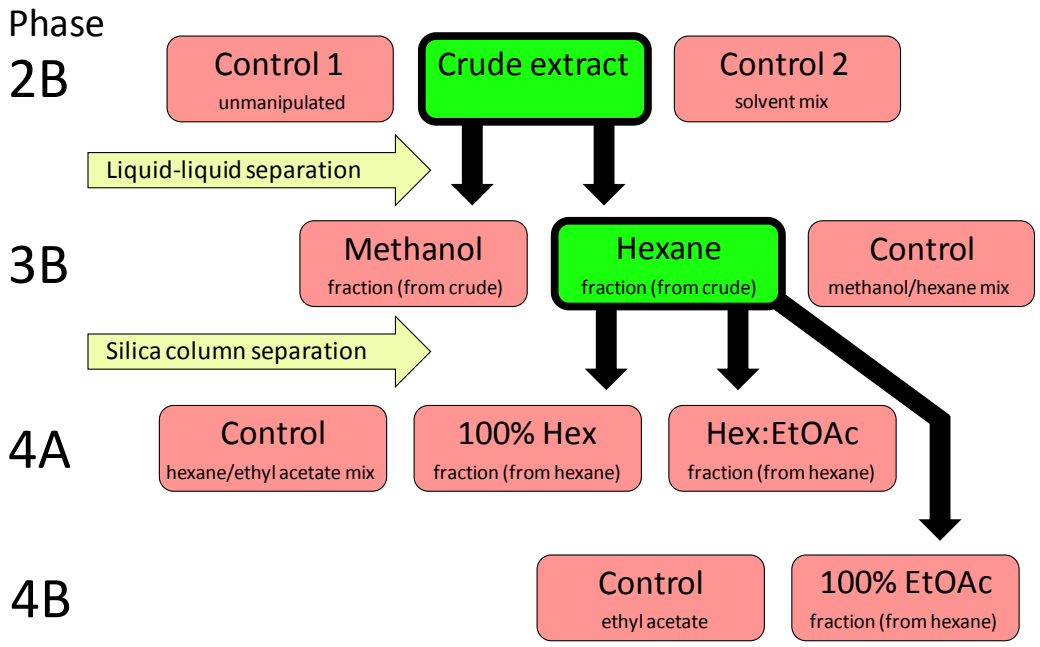

Figure 9. Overview of the bioassay results in phases 2B, 3B, 4A and 4B. Green squares (bold outline) indicate a significant feeding response, while red squares (thin outline) symbolize no snake responses to the test substances or controls. Results with mouse bait in phase 2A and 3A were qualitatively similar to those with eraser bait in phase 2B and 3B, but showed more variation.

Scent Dosages

Despite our focus on compound dry weights applied to the bait matrices, it is of some interest to know roughly how many geckos were needed to produce the extracts. Our extractions and purifications varied in the amounts of extract (in terms of dry weight) we obtained per gecko. Also, the dry weight amount applied to a bait matrix was not identical through all trial phases, and the fractionation of the crude extract obscured how many gecko equivalents were used to produce scent for one bait matrix – especially since some of the more abundant fractions were not used. But in general, one gecko helped scent a handful of baits. We consider this amount of extract rather large, given that snakes can presumably detect the scent trail of geckos that have merely walked over a leaf or along a branch. The latter must undoubtedly offer a much weaker scent than that of our bait matrices. Of course, snakes normally do not try to ingest the leaves and branches geckos have walked over [see Keiser et al. (2011) for a possible exception], but several of the snakes we tested ingested (or tried to ingest) the non-animate bait matrices we had scented

with certain extracts. In hindsight, that indicates to us that the general dosage was sufficiently high to elicit a response.

Chemical Analyses of Scent Extracts

The GC-MS spectrum of the original (as opposed to the sub-fractioned) hexane fraction revealed one dominant peak. Based on the retention time (upper panel of Fig. 10) and the fractionation pattern in the mass spectrum (lower panel of Fig. 10) it was found to be cholesterol.

The sub-fractionation of the hexane fraction (Si-Hex, Si-Hex-DCM, Si-DCM-MeOH) helped characterize other groups of compounds present in the original hexane fraction. These included non-aromatic hydrocarbons (Si-Hex fraction, Fig. 11), minute amounts of aromatic hydrocarbons (Si-Hex-DCM fraction), and heterocompounds (Si-DCM-MeOH) including fatty acids and sterols (Fig. 12, Table 2). Based on retention times (which were correlated to compound mass), type of separation columns used in the GC, and experimental condition (e.g., amount of gas flow, temperature of column), we could identify four unbranched saturated hydrocarbons in the Si-Hex fraction. Many of the mass peaks belonging to this fraction were polymethylated alkanes, based on their mass and fractionation pattern. However, as the fractionation pattern is not conclusive to the position of the methyl groups, no further structural assignment can be conducted without isolating the compounds and conducting additional spectroscopic experiments. Squalene was the only polyunsaturated hydrocarbon that could be identified based on retention time and mass spectra. Squalene is a triterpene and an important part in the biosynthesis of cholesterol. As it is a liquid and volatile, as most hydrocarbons are, it could play a role in the snakes' identification of prey. The main peak of the hydrocarbon fraction (Si-Hex) with retention time 67.34 min (Fig. 11) had a similar retention time as cholesterol (67.40 min, Fig. 10) in the active hexane fraction, but mass data indicated the shown hydrocarbon ($C_{31}H_{64}$). The major compound of the Si-DCM-MeOH was also cholesterol (67.36 min), while smaller amounts of saturated and unsaturated fatty acids were present as well (Fig. 12). Identified saturated fatty acids are summarized in Table 2.

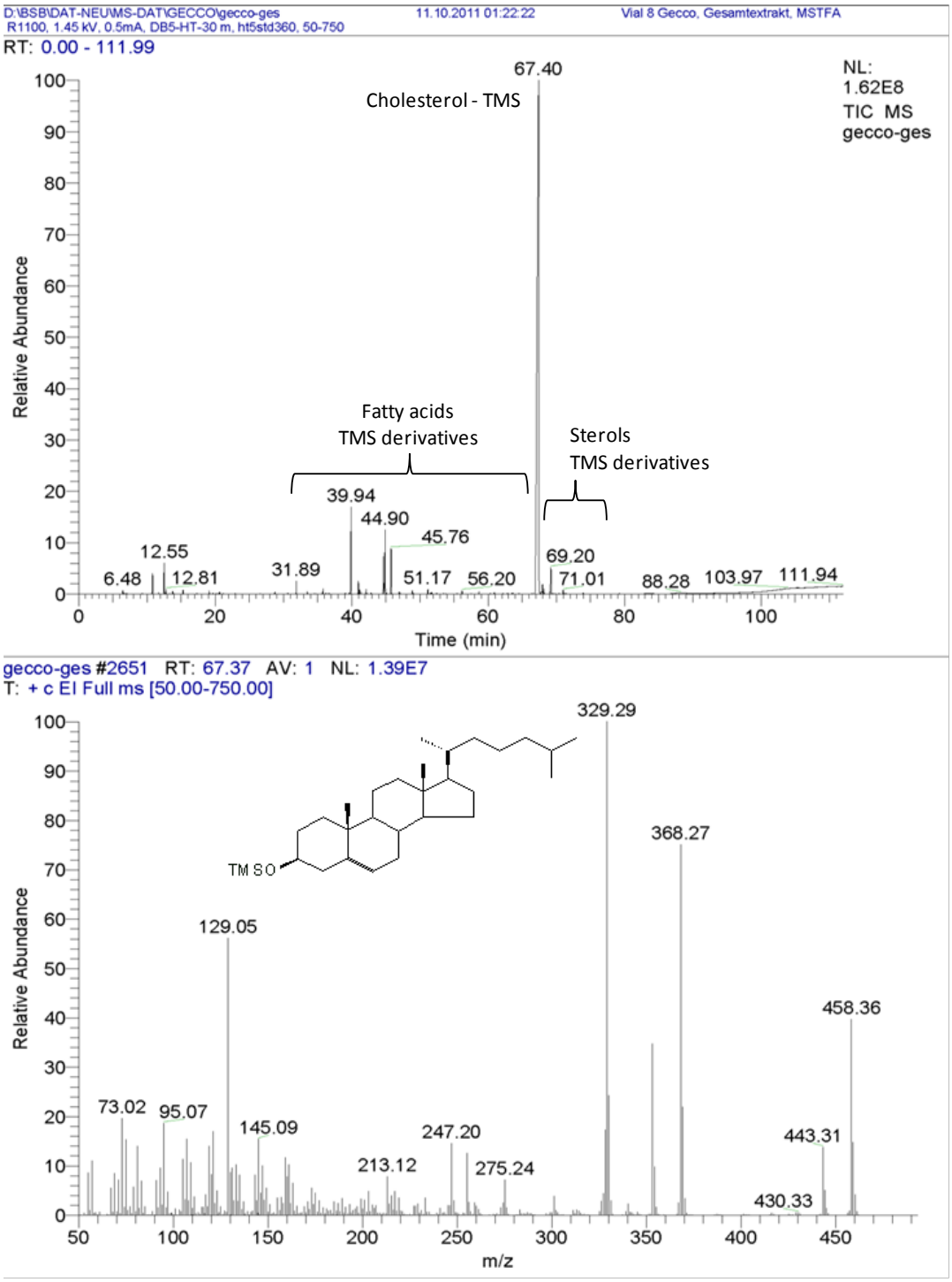

Figure 10. GC-MS chromatogram and corresponding mass spectrum of the active hexane fraction. The chromatogram is dominated by one compound/peak, which shows the typical retention time for cholesterol at 67.4 minutes. The mass spectrum of cholesterol is shown below with the typical fragmentation pattern (molecular weights of the compound fragments on the x-axis) of the cholesterol trimethylsilyl (TMS) derivative.

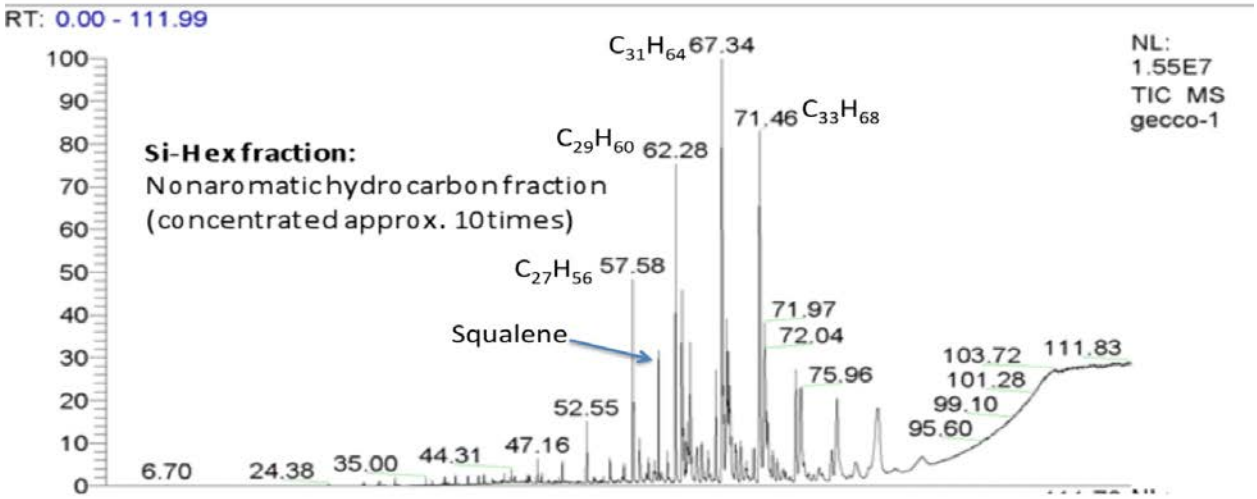

Figure 11: GC-MS chromatogram of the Si-Hex sub-fractionation of the hexane fraction, showing non-aromatic hydrocarbons. Some of the major peaks are identified and molecular formula given.

LC-MS analysis of the active hexane fraction revealed 112 mass peaks (Appendix 1). By cross-referencing the peaks obtained from the active hexane fraction with peaks from the inactive methanol fraction we could identify three compounds that were present solely in the hexane fraction. All other peaks were present in either both extracts or only the inactive methanol extract although in different concentrations. However, since both fractions were analyzed with different ionization modes, which again were different from the GC-MS analysis, some peaks could have been detected by one ionization mode but not the other. The compound could still be present in both fractions, but just not ionize under the experimental conditions. Therefore peaks detected only in the hexane fraction have to be considered with some caution as they may not be unique to the hexane fraction.

The chemical data obtained for the three peaks found only in the active hexane fraction had molecular masses of 278.217300, 282.248600, and 284.264220, suggesting molecular formulae of $C_{18}H_{30}O_2$, $C_{18}H_{34}O_2$, and $C_{18}H_{36}O_2$ respectively. Based on the molecular formulae no further structural assignment could be done. However, samples could be further analyzed with MS/MS analysis followed by interpretation of the resulting mass spectra (which are spectra of the compounds fragments) and additional nuclear magnetic resonance analysis, to identify the chemical structure of the compounds. As we have no information on the activity of the three compounds it would seem a better strategy to continue with fractionation of the hexane extract into different compound classes as carried out during the GC-MS analysis. The next step would then be bioassays with BTS to test which fractions show activity (hydrocarbon Si-Hex or the heterocompound Si-DCM-MeOH fraction).

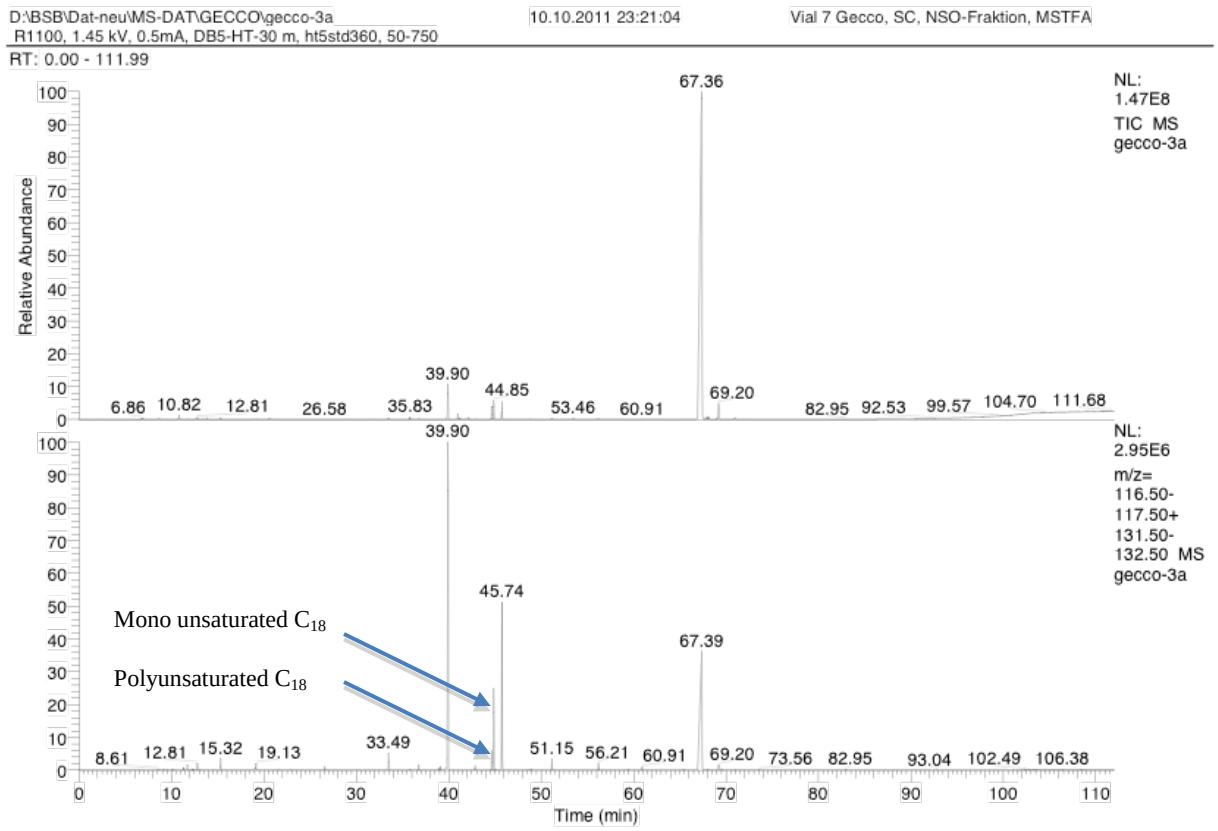

Figure 12. GC-MS of the Si-DCM-MeOH hexane subfraction, showing heterocompounds. The upper chromatogram gives the relative abundance of the peaks to each other, with the peak 67.39 (cholesterol) being the major compound. The lower chromatogram enhanced the less abundant compounds, which are mainly saturated and unsaturated fatty acids.

Table 2. Summary of the identified saturated acids based on retention times and mass spectra.

Retention time	Molecular mass	Molecular formula	Name	State
15.32	158.24	$C_9H_{18}O_2$	Nonanoic acid	crystal
19.13	172.26	$C_{10}H_{20}O_2$	Decanoic acid	crystal
33.50	228.37	$C_{14}H_{28}O_2$	Myristic acid	crystal
39.90	256.42	$C_{16}H_{32}O_2$	Palmitic acid	crystal
45.74	284.48	$C_{18}H_{36}O_2$	Stearic acid	crystal
51.15	312.53	$C_{20}H_{40}O_2$	Arachidic acid,	crystal
56.20	340.58	$C_{22}H_{44}O_2$	Behenic acid	crystal
60.91	368.63	$C_{24}H_{48}O_2$	Lignoceric acid	crystal
67.39	386.65	$C_{27}H_{46}O$	Cholesterol	crystal

Our results demonstrate a proof of concept by showing the attractant properties of the crude extract and the hexane fraction. Although many snakes did not respond to either treatments or controls, when snakes did respond, the majority responded to the treatment with gecko scent rather than to controls. The latter was particularly true when we used bait matrices that were in themselves completely foreign to the snakes (i.e., erasers as opposed to dead neonatal mice). In addition, we could also identify the main compound in the active hexane fraction as cholesterol, as well as several compounds in the hydrocarbon (Si-Hex) and the heterocompound Si-DCM-MeOH fraction. Why we lost the activity of the hexane fraction after the fractionation in phase 4 is not clear. It might be that not only one compound, but several compounds in combination are required to elicit a feeding behavior in BTS.

Conclusion and Implications for Future Research

Our snake bioassays showed that the crude gecko extract, as well as one of the purified fractions resulting from our first separation of the crude extract, contained something that attracted small Brown Treesnakes. With the protocol and dosages we used, the attraction was strong enough to convince some of the snakes – but far from all – that a non-animate object we tainted with the crude extract or the derived hexane fraction was edible (i.e., a 'legitimate' prey). The fact that some snakes started chewing on the tainted eraser bait matrices but then spat them out suggests the texture of these particular matrices may have caused the snakes to change their perception about their suitability as food.

The chemical analyses that followed showed cholesterol to be the prime candidate for the attraction effect, as it was the most abundant compound in the hexane extract. Other compounds identified from the hydrocarbon (Si-Hex) and heterocompound (Si-DCM-MeOH) fractions were different length alkanes and saturated and unsaturated fatty acids, respectively. The GC-MS analysis proved useful in identifying the major compounds and continued chemical analysis (e.g., isolation of compounds and spectroscopic analysis) should allow identification of the remaining compounds present. One strategy for future research would be to test the hydrocarbon and heterocompound fractions each as a whole. Depending on which of the fractions exhibits activity in soliciting feeding responses in BTS, further chemical analysis could focus on that fraction, and identify the major components. One reason the snakes failed to respond in Phase 4 could possibly be that the scent of several compounds in conjunction are required to trigger a feeding response. In this case one might need to use several of the identified compounds combined to get the attractant effect. Ultimately field tests would be needed to see if bioassay results from the lab are transferable. However as there are many unidentified polymethylated alkanes in this fraction as well, it would be best to test this fraction as a whole for feeding activity with BTS (see recommendation).

Because we do not yet know the identity of all compounds in the bioactive fraction, nor if any compound(s) other than cholesterol would be a necessary component, we cannot estimate what the operational control cost for a snake attractant would be. Since most of the compounds seem to be rather structurally simple molecules, we suspect that many (or even most) of them are commercially available compounds. The fatty acids and alkanes we have identified so far (Table 2, Figure 11, 12) are all commercially available. That would help circumvent the need for complicated and costly development of synthesis methods. Should cholesterol alone be sufficient, the cost is low (currently $290 per 500g at a major chemical supplier) given the small amounts necessary to attract a snake (2 mg of the fraction containing cholesterol resulted in a feeding response in several snakes). If additional cues are among the hydrocarbons or heterocompounds seen in the GC-chromatograms (Fig. 10, 11, 12), it is likely the compounds are commercially available, and at a price which would make baiting with chemicals economically feasible.

Small Snake Attractant in Operational Control

We can envision two different uses for a small snake attractant in operational control. Future research would have to be tailored in accordance to which of these uses is the goal, unless both are used in conjunction.

One use would be to put gecko scent on baits that had been inserted with an oral toxicant; thereby helping to kill the snake by overcoming the apparent hesitation of the small snakes to ingest rodent baits currently used in operational control. We will term this ***proximate*** control use. We currently do not know if a higher scent dosage might have helped increase the proportion of snakes that responded positively and unambiguously to the scent-treated baits. We also do not know to what extent the snakes might use visual information when deciding whether or not to grasp and ingest a potential food item. If that is the case, applying the scent to a more natural-looking (i.e., lizard-like) object may render a stronger and more uniform (across snake individuals) response. We note the somewhat surprising observation that un-scented plastic lizards have been ingested by Brown Treesnakes trapped in a field study (Savarie and Clark 2006) suggesting that visual cues also may play a role in prey selection. These questions suggest that a future study (with a candidate attractant) should test dosage dependence as well as alternative bait matrices.

Another control use of a small snake attractant would be to help lead snakes up to (and into) a control device (i.e., a trap or a bait station). We term this the ***ultimate*** control use. In this case dosage dependence is still of some interest, but the issue of finding a bait matrix with a suitable texture and/or look does not apply.

The current state of knowledge does not allow us to say for certain whether the ultimate or the proximate use would be the most important aspect to help improve control of small snakes. We (the USGS-CSU Brown Treesnake Project) have just started investigating movements and prey approach strategies in small Brown Treesnakes, results of which should improve our understanding of which opportunities and obstacles may be associated with the ultimate aspect of attractant use to aid snake control. Of course, it would be ideal if an attractant could be used to improve snake control via *both* the ultimate and the proximate aspects.

Future Research Needs

Given that we could demonstrate a proof of concept by identifying compounds which elicited a feeding response in small BTS in fractions from gecko skin extracts, we suggest that further research is needed. Having identified some of the major compounds in the bioactive hexane fraction (cholesterol, fatty acids, alkanes) and being able to establish what the major compound classes are (e.g., fatty acids, alkanes), the next step could be bioassays to identify which of the major compound classes is the bioactive fraction (e.g., fatty acids or alkanes). Once the bioactive

fraction has been identified a detailed chemical study identifying all the components of the bioactive fraction needs to be done. Once this has been achieved, testing of groups of compounds and pure compounds (most of which should be commercially available) in the laboratory and later in the field should be carried out to identify the chemical(s) responsible for the feeding response. Testing candidate feeding attractants in laboratory and field assays could be a separate study or done in conjunction with the chemistry work.

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Appendix A.

Measured molecular weight of compounds identified in the hexane and methanol fraction of the Gecko extracts

The following table is a list of the measured molecular weight of compounds identified in the hexane and methanol fraction of the Gecko extracts (step 3B in Figure 9). Only compounds that were present in higher quantities in the hexane or methanol fraction (peaks with intensities over 800,000,000) were considered. Based on the measure fine mass, the analytical software (Bruker Solarex) was able to calculate the molecular formulae. No reference refers to the case where several molecular formulae would be possible based on the measured mass. The compounds in bold font indicate molecular weights which were present only in the active hexane fraction and not the inactive methanol fraction. **m/z**: mass to charge ratio; **APCI_neg-Hexane**: hexane fraction was analyzed using atmospheric-pressure chemical ionization in negative mode (eliminating one proton form the molecule); **ESI$^-$ MeOH**: methanol fraction was analyzed using electrospray ionization in negative mode (eliminating one proton from the molecule).

Measured molecular weight_m/z	Molecular Formula	Peak Intensity	APCI_neg-Hexane	ESI_neg-MeOH
254.080100	no reference	928714624		928714624
277.217300	**$C_{18}H_{30}O_2$**	**885560209**	**13512120**	
279.038900	no reference	2576964000		2576964000
279.232933	$C_{18}H_{32}O_2$	1557592255	50318488	12781885
281.036000	no reference	847994560		847994560
281.248600	**$C_{18}H_{34}O_2$**	**2479024336**	**269396928**	
283.264220	**$C_{18}H_{36}O_2$**	**1795744578**	**761416448**	
303.050200	no reference	1328391040		1328391040
337.129275	$C_{17}H_{22}O_7$	1190138496	831096128	305792864
339.144924	$C_{17}H_{24}O_7$	997861824	383557632	240487776
341.124190	$C_{16}H_{22}O_8$	1174159360	319994304	255857472
351.108539	$C_{17}H_{20}O_8$	1134603264	956052480	284307360
351.144926	$C_{18}H_{24}O_7$	1073785984	789722240	269627328
353.124188	$C_{17}H_{22}O_8$	1663353088	794184512	420754720
355.103452	$C_{16}H_{20}O_9$	1066194176	325542240	263404160
355.139840	$C_{17}H_{24}O_8$	1177867648	350970336	285227680
363.108539	$C_{18}H_{20}O_8$	829629312	1038923456	203542400
365.160575	$C_{19}H_{26}O_7$	887043840	695257984	214226096
367.103453	$C_{17}H_{20}O_9$	1160857856	702996096	315513248
367.139836	$C_{18}H_{24}O_8$	1631930752	935649024	431249824
369.155489	$C_{18}H_{26}O_8$	928340992	337506912	245456544
371.134753	$C_{17}H_{24}O_9$	899771904	222168336	239231872
377.124190	$C_{19}H_{22}O_8$	1071751872	1115765504	271741376
379.103456	$C_{18}H_{20}O_9$	1118417920	863849536	286422624
379.139838	$C_{19}H_{24}O_8$	1817293824	1327320192	461141632
381.155489	$C_{19}H_{26}O_8$	1696187776	870145408	416840480
383.098368	$C_{17}H_{20}O_{10}$	1046074816	389377408	259162000

Measured molecular weight_m/z	Molecular Formula	Peak Intensity	APCI_neg-Hexane	ESI_neg-MeOH
383.134753	$C_{18}H_{24}O_9$	1991890432	735800768	486178720
385.114015	$C_{17}H_{22}O_{10}$	954852992	262831552	244155408
385.150402	$C_{18}H_{26}O_9$	872539840	248675856	213943344
391.139841	$C_{20}H_{24}O_8$	1066538816	1085385728	255037776
393.119105	$C_{19}H_{22}O_9$	1536567040	1084603008	374642080
393.155490	$C_{20}H_{26}O_8$	1506158336	1016183552	363435424
395.098371	$C_{18}H_{20}O_{10}$	1054488192	495831264	275356096
395.134754	$C_{19}H_{24}O_9$	2341615360	1155123456	588028352
395.171140	$C_{20}H_{28}O_8$	1182414592	566872448	292723168
397.114018	$C_{18}H_{22}O_{10}$	1858225664	630445824	470588928
397.150405	$C_{19}H_{26}O9$	1974617728	714463040	504667680
399.129672	$C_{18}H_{24}O_{10}$	1509840256	411428064	364290624
407.134754	$C_{20}H_{24}O_9$	1484165504	1137842304	385135200
407.171142	$C_{21}H_{28}O_8$	933925376	689182912	234533504
409.114019	$C_{19}H_{22}O_{10}$	1560453120	780281024	412268096
409.150406	$C_{20}H_{26}O_9$	1995612288	1034560768	508737120
411.129672	$C_{19}H_{24}O_{10}$	2260905728	804925632	582007360
411.166053	$C_{20}H_{28}O_9$	1460580096	533344512	371243616
413.108936	$C_{18}H_{22}O_{11}$	968015168	244989008	256491024
413.145318	$C_{19}H_{26}O_{10}$	1578286464	426261632	410041888
419.134757	$C_{21}H_{24}O_9$	849274880	975330816	215861632
421.114019	$C_{20}H_{22}O_{10}$	1121646080	767715456	267570480
421.150404	$C_{21}H_{26}O_9$	1574630912	1139697792	371641664
423.129667	$C_{20}H_{24}O_{10}$	2237858816	1007842048	524865760
423.166054	$C_{21}H_{28}O_9$	1789068416	835613504	406114560
425.108932	$C_{19}H_{22}O_{11}$	1328747008	411204896	331207808
425.145320	$C_{20}H_{26}O_{10}$	2492666368	829586752	593417344
425.181704	$C_{21}H_{30}O_9$	1063457408	371883392	253678768
427.124580	$C_{19}H_{24}O_{11}$	1423908864	368346944	354539552
427.160971	$C_{20}H_{28}O_{10}$	1372528640	385910624	343398464
435.166055	$C_{22}H_{28}O_9$	1152079616	832220544	303031936
437.108930	$C_{20}H_{22}O_{11}$	1093887104	520664608	296544736
437.145321	$C_{21}H_{26}O_{10}$	2203280640	1128969856	581757440
437.181705	$C_{22}H_{30}O_9$	1140548864	620017280	299362816
439.124584	$C_{20}H_{24}O_{11}$	2029744640	711114496	545320320
439.160969	$C_{21}H_{28}O_{10}$	2272489984	832749376	599715200
441.140236	$C_{20}H_{26}O_{11}$	2010873728	526567392	496562400
441.176619	$C_{21}H_{30}O_{10}$	1217363968	321308672	307032288
443.155885	$C_{20}H_{28}O_{11}$	990350720	196202624	231732304
449.145322	$C_{22}H_{26}O_{10}$	1199027712	851634496	300482144
451.124580	$C_{21}H_{24}O_{11}$	1323549568	657256768	339608000
451.160970	$C_{22}H_{28}O_{10}$	1685308416	896201024	428278208

Measured molecular weight_m/z	Molecular Formula	Peak Intensity	APCI_neg-Hexane	ESI_neg-MeOH
453.140237	$C_{21}H_{26}O_{11}$	1999884672	761985280	513344800
453.176617	$C_{22}H_{30}O_{10}$	1479790976	582285632	377816352
455.119502	$C_{20}H_{24}O_{12}$	1150279424	300613824	297273440
455.155883	$C_{21}H_{28}O_{11}$	1745084160	494469344	443222112
457.135148	$C_{20}H_{26}O_{12}$	939781312	205719680	240192432
463.160967	$C_{23}H_{28}O_{10}$	1102583296	752819968	288363904
465.140234	$C_{22}H_{26}O_{11}$	1657021440	751511104	416880832
465.176619	$C_{23}H_{30}O_{10}$	1476142080	684926528	363141344
467.119501	$C_{21}H_{24}O_{12}$	1362637184	377302368	328932352
467.155885	$C_{22}H_{28}O_{11}$	2193895936	710094208	526982144
467.192268	$C_{23}H_{32}O_{10}$	1148989952	377433504	267918352
469.135146	$C_{21}H_{26}O_{12}$	1669087104	375076000	395255616
469.171535	$C_{22}H_{30}O_{11}$	1621377024	416363712	378609472
471.150794	$C_{21}H_{28}O_{12}$	1135365632	225655744	269099616
477.176622	$C_{24}H_{30}O_{10}$	807696384	560025792	185084928
479.119503	$C_{22}H_{24}O_{12}$	855147904	347297600	189148928
479.155884	$C_{23}H_{28}O_{11}$	1479837184	691754816	325857056
479.192271	$C_{24}H_{32}O_{10}$	964462080	466704256	209465120
481.135150	$C_{22}H_{26}O_{12}$	1582076800	508124672	338899488
483.150801	$C_{22}H_{28}O_{12}$	1789174144	471950528	392770880
483.187189	$C_{23}H_{32}O_{11}$	1183359488	325412064	258553168
485.166449	$C_{22}H_{30}O_{12}$	1140109312	246836048	250361952
493.135149	$C_{23}H_{26}O_{12}$	1052567232	392135904	232604768
493.171542	$C_{24}H_{30}O_{11}$	1294526208	548766976	283722848
495.150800	$C_{23}H_{28}O_{12}$	1719465088	511150880	360269664
495.187191	$C_{24}H_{32}O_{11}$	1357182080	432376640	279594848
497.130061	$C_{22}H_{26}O_{13}$	1104740864	232165712	231098944
497.166451	$C_{23}H_{30}O_{12}$	1740964352	410227040	354765376
497.202840	$C_{24}H_{34}O_{11}$	821887552	215519616	169953872
499.145714	$C_{22}H_{28}O_{13}$	1028984704	184326016	209145120
499.182100	$C_{23}H_{32}O_{12}$	967380928	191731600	192564528
507.150799	$C_{24}H_{28}O_{12}$	1063601216	447983456	213932144
507.187188	$C_{25}H_{32}O_{11}$	997803072	457027456	198662256
509.130059	$C_{23}H_{26}O_{13}$	893997248	263501104	184900384
509.166445	$C_{24}H_{30}O_{12}$	1488802048	507753824	308828960
509.202839	$C_{25}H_{34}O_{11}$	891375936	310883680	188046128
511.145710	$C_{23}H_{28}O_{13}$	1202019712	302693120	266558928
511.182092	$C_{24}H_{32}O_{12}$	1301896704	364624672	281828832
513.161364	$C_{23}H_{30}O_{13}$	987589248	211861712	219111552
514.284400	$C_{26}H_{45}O_{7}N_{1}S_{1}$	2208465000		2208465408
521.166450	$C_{25}H_{30}O_{12}$	832412416	356112192	202926800
523.145710	$C_{24}H_{28}O_{13}$	900310720	278322368	212430144

Measured molecular weight_m/z	Molecular Formula	Peak Intensity	APCI_neg-Hexane	ESI_neg-MeOH
523.182098	$C_{25}H_{32}O_{12}$	1096394496	361749696	259091792
525.161360	$C_{24}H_{30}O_{13}$	1141486208	289727040	265056176
525.197745	$C_{25}H_{34}O_{12}$	901362304	253682256	206663616
527.177004	$C_{24}H_{32}O_{13}$	896646592	190572448	198472208
537.161362	$C_{25}H_{30}O_{13}$	829027008	321126880	187137360
539.177013	$C_{25}H_{32}O_{13}$	909769920	291702560	216629104